# Characterization of *Pseudomonas aeruginosa* from cystic fibrosis lung infections

## Introduction

*P. aeruginosa* is an opportunistic pathogen that causes a range of acute diseases in both humans and animals due to its large number of virulence genes which directly interfere with different hosts and their defence systems. One of the best studied human infections caused by *P. aeruginosa* is airway infections in patients suffering from the genetic disorder cystic fibrosis (CF). Due to a severely reduced mechanical clearing of the airways in these patients they acquire multiple infections of various bacteria and fungi, but the major cause of morbidity and mortality is chronic infection of *P. aeruginosa* in the lungs. The development of chronic lung infections follows a characteristic pattern (Figure 1). Typically, a period of intermittent colonization with *P. aeruginosa* early in the life of the patient precedes the establishment of chronic infections. These early-infecting strains typically resemble those found in the environment. This stage is temporary, and eventually the airways of nearly all patients become permanently colonized by *P. aeruginosa*. Once established, these chronic lung infections are difficult to eradicate even with intensive antibiotic treatment.

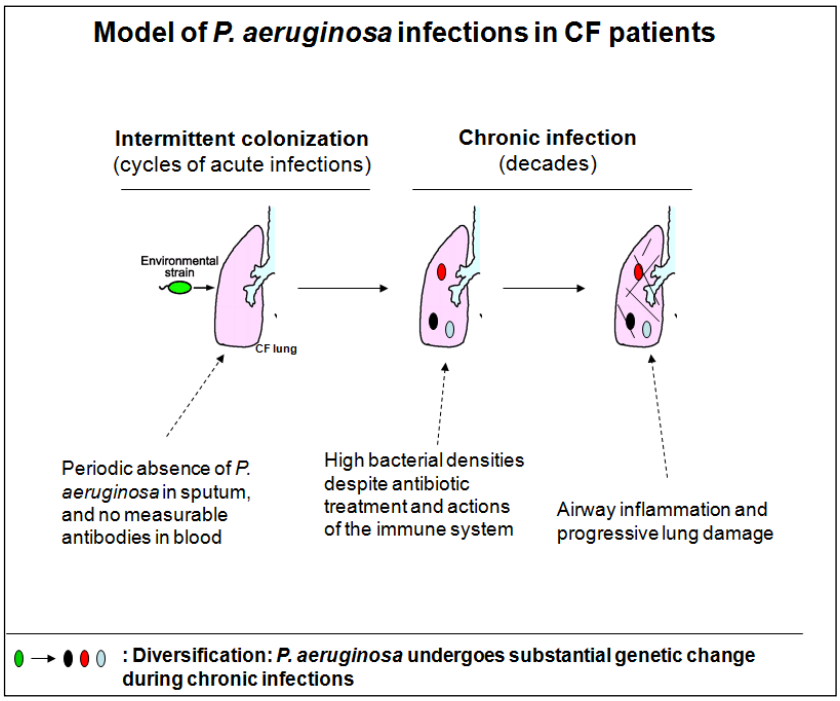



***Figure 1.*** *Model of Pseudomonas aeruginosa infections in cystic fibrosis patients.*

At the molecular level, the development of chronic infections have been described as multi-stage colonization processes which initially require the encoded virulence properties of the bacteria to establish a successful colonization followed by switching to a less virulent expression mode controlled global transcriptional regulators in *P. aeruginosa*, which direct the bacterial population towards a chronic infection. At the same time, the infection process in the CF airways is associated with extensive genetic adaptation and microevolution of the infecting bacteria: During the long-term chronic infection stage the bacteria gradually loose the function of many genes associated with bacterial pathogenicity. The accumulation of mutations results in genetic variants with

phenotypes of which many are not usually observed among environmental isolates. These phenotypes include loss of motility, loss of effector proteins of the type III secretion system, reduced virulence, and increased antibiotic resistance. In some cases, specific genes have been found to be hot spots for mutations during CF infections. These common targets include *lasR*, which encodes a quorum-sensing regulator, and *mucA*, which results in the overproduction of alginate and conversion to the frequently found mucoid phenotype.

**Description of the exercise**

As a part of our research on *P. aeruginosa* infections, we have been following various CF patient groups and their infecting *P. aeruginosa* strains for several years (See supplementary papers).

In this exercise, each team will analyze 1 isolate of *P. aeruginosa* strains sampled from a CF patient that is of particular interest to us. All isolates that we will analyze in this exercise are from the same host but different years (longitudinal samples). We know very little of the isolates but on the basis of genotyping and relevant phenotype assays performed on these isolates each group will make new contributions to our understanding of the infection dynamics in this particular patient.

*Experimental keywords: genotyping using SNP arrays, infection dynamics analysis, antibiotic resistance profiling.*

**Relevant references:**

Lars Jelsbak, Helle Krogh Johansen, Anne-Louise Frost, Regitze Thøgersen, Line Elnif Thomsen, Oana Ciofu, Lei Yang, Janus Haagensen, Niels Høiby, Søren Molin. (2007). ***Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients.*** Infect Immun. 75(5):2214-24.

Yang L, Jelsbak, L, Marvig RL, Damkiær S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu, O, Høiby N, Sommer MO, and Molin S. 2011. ***Evolutionary dynamics of bacteria in a human host environment*****.** *Proc Natl Acad Sci USA*. doi: 10.1073/pnas.1018249108

Martin Holm Rau, Susse Kirkelund Hansen, Helle Krogh Johansen, Line Elnif Thomsen, Christopher T. Workman, Kristian Fog Nielsen, Lars Jelsbak, Niels Høiby, Lei Yang and Søren Molin. (2009). ***Early adaptive developments of Pseudomonas aeruginosa after the transition from life in the environment to persistent colonization in airways of human cystic fibrosis hosts*****.** Environ Microbiol. 2010 Jun;12(6):1643-58.

Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, Köhler T, van Delden C, Weinel C, Slickers P, Tümmler B. (2007) ***Population structure of Pseudomonas aeruginosa.*** Proc Natl Acad Sci U S A. 104(19):8101-6.

Count

| | Pseudomonas genotyping | Antibiotic resistance |
|---|---|---|
| Day 1 | Multiplex PCR for AT chip | Prepare colistin plates and inoculate strains |

**End of the day results:** 1 PCR stored at min 4°C, 2 colistin prepared LB plates, control (PAO1) and isolate strain in liquid LB (37°C shaking).

| | | |
|---|---|---|
| Day 2 | Hybridization and detection | Antibiotic susceptibility testing by disk diffusion |

**End of the day results:** Pictures of AT-chip results, 4 LB plates with bacteria and antibiotics incubating at 37°C.

| | | |
|---|---|---|
| Day 3 | Data analysis | Inspect plates and record results - Incubate again- |

**End of the day results:** Pictures of 4 LB plates with bacteria and antibiotics, and notes of antibiotic susceptibility/resistance of strain(s).

| | | |
|---|---|---|
| Day 4 | | Inspect plates and record results |

**End of the day results:** Pictures of LB plates with bacteria and antibiotics, and notes of antibiotic susceptibility/resistance of strain(s).

**Experimental procedures**

DAY 1

**(A) *P. aeruginosa* genotyping**

It is important that you have an understanding of the ArrayTube genotyping method before we start the experiments. Please consult appendix 3 and also the supplementary papers for this exercise – in particular the paper by Wiehlmann et al (2007).

***Note: AT chip genotyping is performed on both isolates in independent reactions. The following protocol is described for 1 reaction***

Preparation of bacteria

1. Swipe 2 loops of bacteria off the agar plate and resuspend in 100µl $H_20$ in an eppendorf tube.
2. Heat for 5min in a heating block at 99C
3. Cool on ice before using 5µl of the suspension for your PCR reaction (see next step)

Preparation of the PCR reaction:

1. Prepare a Master Mix by combining 4.9 µl of B1 (2X Labelling buffer) and 0.1 µl of B2 (DNA polymerase) per specimen.
2. Mix the following in a PCR tube:
   5 µl DNA suspension
   5 µl Master Mix.

**2.** Amplification protocol:

| | |
|---|---|
| Initial denaturation 96°C 300 s | |
| 50 cycles with | 20 sec at 62°C |
| | 40 sec at 72°C |
| | 60 sec at 96°C |
| Cool down and store at 4°C | |

**(B) Antibiotic susceptibility testing using Disk Diffusion**

Preparation for tomorrow:

1. Inoculate a single colony of each isolate in 10ml LB medium and incubate at 37C ON.
2. Inoculate a single colony of *Pseudomonas aeruginosa* PA01 (reference strain) in 10ml LB medium and incubate at 37°C ON

3. We need to prepare colistin plates for "Antibiotic susceptibility testing using Disk Diffusion" tomorrow (Read appendix 1 to learn why):
    - Label an LB agar plate for each of your isolates
    - Place a colistin tab ("CO150") on each plate using forceps
    - After 2 hours at room temperature the tablet is removed (by knocking the plate against the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).
    - Outline the position of the tab with a pen on the backside of the agar plate.

DAY 2

**(A) *P. aeruginosa* genotyping**

****Important notes – read the following before you start the experiment***:*
*- Do not centrifuge ArrayTubes (AT)*
*- Do not scratch or touch the array surface (e.g. during pipetting steps with the pipette tip)*
*- Avoid formation of air bubbles and remove them, if necessary, by mixing with a pipette*
*- Never let the AT run dry*

Preparation of the hybridisation mixture
Prior to hybridization, the AT array needs to be conditioned:

1. Pre-heat the thermomixer to 60°C
2. Add 90 µl of buffer C1 to each PCR tube, mix gently (vigorous mixing results in foaming) and put aside.

Pre-washing of the arrays (2 washing steps)

1. Remove the ArrayTube from the bag
2. Add 500 µl of ultrapure water to each tube
3. Incubate in the thermomixer at 60°C, 550 rpm for 2 minutes
4. Remove and discard the water WITHOUT TOUCHING THE ARRAY SURFACE
5. Add 200 µl buffer C1 to each tube
6. Incubate in the thermomixer at 60°C, 550 rpm for 2 minutes
7. Remove at discard buffer C1 **(hybridisation mixtures must be ready when C1 is removed)**
8. Proceed promptly (hybridisation mixtures must be ready when C1 is removed)

Hybridization.

1. Transfer each hybridisation mixture (100 µl) to a prepared ArrayTube (avoid extensive foaming)
2. Incubate for **one hour** at 60°C, 550 rpm on the thermomixer

**Meanwhile** start computer, AT Reader and IconoClust-AT recording software.

Washing after Hybridisation

1. Remove the ArrayTubes from the thermomixer and set the thermomixer to 30°C for the following steps.
2. Remove *hybridization mix* from the AT as completely as possible (without touching the surface)
3. Add 500 µl of buffer C2
4. Remove and discard the washing solution
5. Add 500 µl of buffer C2 and incubate in the thermomixer for 5 min at 30°C, 550 rpm
6. Remove the buffer and repeat the last washing step (step 5).

Staining and detection.

1. Combine reagent C3 (HRP) and buffer C4 = 1: 100 => C3/C4
   For one sample mix 1 µl C3 with 100 µl C4.
2. Add 100 µl of C3/C4 to each tube
3. Incubate for 10 min at 30°C and 550 rpm on the thermomixer
4. Remove and discard C3/C4
5. Add 500 µl of buffer C5
6. Remove and discard and repeat step.

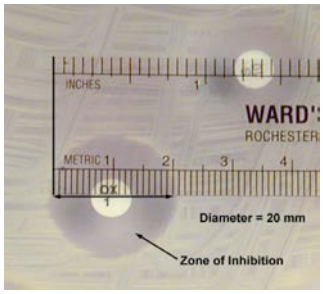
Image acquisition

1. Add 100 µl of reagent D1. Immediately insert the AT inside the AT reader and start image acquisition (note: the TMB-staining process is usually finished after 10 min).

Notes:
- Take more than just 1 image.
- The outside of the AT may be gently (!) cleaned using a piece of lens-paper with ethanol
- If there is "dirt" inside the AT, then remove the TMB solution (and the dirt) after you are done with the image acquisition, add 100µl of hybridization solution and acquire an extra image.

**(B) Antibiotic susceptibility testing using Disk Diffusion**

Susceptibility testing is of great importance to the hospital physician and the practitioner in the treatment of infectious diseases. Knowledge of susceptibility to different antibiotics of an infectious agent facilitates the choice of the most effective antibacterial compounds. In most clinical laboratories the *disk diffusion method* is used routinely for testing the susceptibility of bacterial pathogens. In this part of the exercise, you will test the susceptibility of your isolates to different clinically relevant antibiotics: Gentamycin, Enrofloxacin, Tobramycin and Polymyxins (*a.k.a.* colistin) (see the table below for the disks used in this exercise).




An example of antibiotic susceptibility testing using the Disk Diffusion method. You measure the diameter of zones of growth inhibition in millimeters. The zone seen in this example measures 20 mm in diameter.

| Product | Code | Potency |
|---|---|---|
| Gentamycin | GEN40 | 40 µg |
| Gentamycin | GN250 | 250 µg |
| Enrofloxacin | ENROF | 10 µg |
| Polymyxins (Colistin) | CO150 | 150 µg |
| Tobramycin | TOB10 | 10 µg |

1. Dry 1 LB plate per culture for 10 min at 37°C
2. Dilute each ON culture 1000x in 0.9% NaCl and spread 100µl on an LB agar plate using a sterile "drigalski spatula".
3. Place the 3 tabs on the agar plates using forceps
4. Incubate the plates at 37° C ON.

DAY 3

**(B) Antibiotic susceptibility testing using Disk Diffusion**

Inspect plates and record results in the data sheets on the following pages. If no growth is observed incubate again ON.

**(A) *P. aeruginosa* genotyping**

Data analysis

For the data analysis you need the following items:

- The acquired images from your AT experiment
- The document "Short manual for analysis of AT chip data" (Appendix 5)
- The Excel sheet "AT chip skabelon" which can be found at the course web-site: *Supplementary Manuals/AT chip manuals and data analysis*.

At the course website you may also find an example of the data analysis

DAY 4

**(B) Antibiotic susceptibility testing using Disk Diffusion**

Inspect plates and record results in the data sheets on the following pages.

**Group:**

Results: Antibiotic susceptibility testing using Disk Diffusion

| Name or number of Clinical isolate | GEN40 | | GN250 | | ENROF | | CO150 | | TOB10 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Measured Zone diameter in mm | S, I, or R ? [(1)] | Measured Zone diameter in mm | S, I, or R ? [(1)] | Measured Zone diameter in mm | S, I, or R ? [(1)] | Measured Zone diameter in mm | S, I, or R ? [(1)] | Measured Zone diameter in mm | S, I, or R ? [(1)] |
| Day 1 | | | | | | | | | | |
| Day2 | | | | | | | | | | |

*Control:*

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| PAO1 (Day 1) | | | | | | | | | | |
| Day 2 | | | | | | | | | | |

[(1)] The isolate is **S**(sensitive), **I**(Intermediate) or **R**(resistant) toward the tested antibiotics. See "Zone diameter Interpretation Table" in appendix 2.

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### Performance of Susceptibility Testing



**Pre-diffusion Method (2 + 18 or 2+22 hours) for Antimicrobials Diffusing Poorly on Agar**

High molecular weight antimicrobials (vancomycin, teicoplanin, daptomycin, colistin) diffuse poorly on agar media, resulting in difficult to interpret results when using the disc diffusion method. Rosco Diagnostica has developed a 2 + 18 or 2+22 hour prediffusion technique, permitting an easier differentiation between susceptible and resistant strains when testing against these antimicrobials.

**Procedure**

One Neo-Sensitabs of the antimicrobial to be tested is placed on an uninoculated plate containing the susceptibility test medium (Mueller-Hinton plain or BHI Agar + 5 % blood).
After 2 hours at room temperature (≤25°C) the tablet (disc) is removed (by knocking the plate agianst the table) and the plate is maintained at room temperature for further 18 or 22 hours (overnight).

The plate is now inoculated with the strain to be tested using a McFarland 0.5 inoculum.

Additional antibiotic discs (Neo-Sensitabs) may be added now using a dispenser (if MH agar is used) and thereafter the plate is incubated overnight at 35-37 °C.

The zones of inhibition are then measured. Zone breakpoints are tentative and for research use only.

**Notice:** In the laboratory, the prediffusion plate can be prepared the day before it is inoculated, in which case there is no loss of time and results are obtained within 24 hours.

## NEO-SENSITABS™
### Zone Diameter Interpretation Tables



| | NEO-SENSITABS | POTENCY | CODE | Zone diameter in mm S | Zone diameter in mm I | Zone diameter in mm R | Break-points MIC µg/ml S | Break-points MIC µg/ml R |
|---|---|---|---|---|---|---|---|---|
| a) q) | Nalidixan (U) | 130 µg | NALID | ≥ 25 | 24-21 | ≤ 20 | ≤ 8 | ≥ 32 |
| | Enterobacteriaceae | | | - | - | < 25 | Reduced susceptibility to quinolones | |
| | Neomycin* | 120 µg | NEOMY | ≥ 25 | 24-21 | ≤ 20 | ≤ 6 | ≥ 25 |
| | Netilmicin | 40 µg | NETIL | ≥ 20 | 19-17 | ≤ 16 | ≤ 12 | ≥ 32 |
| a) | Nitrofurantoin (U) | 260 µg | NITRO | ≥ 23 | 22-20 | ≤ 19 | ≤ 32 | ≥ 128 |
| a) q) | Norfloxacin (U) | 10 µg | NORFX | ≥ 16 | 15-14 | ≤ 13 | ≤ 4 | ≥ 16 |
| i) *) | Novobiocin | **5 µg** | NOVO5 | ≥ 16 | 15-14 | ≤ 13 | ≤ 2 | - |
| q) | Ofloxacin | 10 µg | OFLOX | ≥ 18 | 17-15 | ≤ 14 | ≤ 2 | ≥ 8 |
| | *Staphylococcus* spp. | | | ≥ 20 | 19-17 | ≤ 16 | ≤ 1 | ≥ 4 |
| c) | Oxacillin | **1 µg** | OXA.1 | | | | | |
| | *S. aureus* | | | ≥ 13 | 12-11 | ≤ 10 | ≤ 2 | ≥ 4 |
| | Coag. neg. staph. | | | ≥ 18 | - | ≤ 17 | ≤ 0.25 | ≥ 0.5 |
| a) | Oxolinic acid* (U) | 10 µg | OXOLI | ≥ 16 | 15-14 | ≤ 13 | ≤ 4 | ≥ 8 |
| | Penicillin Low | 5 µg | PEN.L | ≥ 26 | 25-23 | ≤ 22 | | |
| b) | *Staphylococcus* spp. | | | ≥ 26 | - | < 26 | ≤ 0.1 | Beta-Lactamase |
| e) | *Enterococcus* spp. | | | ≥ 10 | - | - | - | ≥ 16 |
| b) | Piperacillin | 100 µg | PIPRA | ≥ 23 | 22-20 | ≤ 19 | ≤ 16 | ≥ 128 |
| h) | *Pseudomonas* spp. | | | ≥ 18 | - | < 18 | ≤ 64 | ≥ 128 |
| d) | Piperacillin+Tazobactam | 100+10µg | PI+TZ | ≥ 23 | 22-20 | ≤ 19 | ≤ 16/4 | ≥ 128/4 |
| h) | *Pseudomonas* spp. | | | ≥ 18 | - | < 18 | ≤ 64/4 | ≥ 128/4 |
| s) | Polymyxins* (colistin) | 150 µg | CO150 | ≥ 20 | 19-17 | ≤ 16 | ≤ 2 | ≥ 4 |
| | Pristinamycin* | 30 µg | PRIST | ≥ 23 | 22-20 | ≤ 19 | ≤ 2 | ≥ 8 |

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### Zone Diameter Interpretation Tables

Enterobacteriaceae

| | NEO-SENSITABS | POTENCY | CODE | Zone diameter in mm S | Zone diameter in mm I | Zone diameter in mm R | Break-points MIC µg/ml S | Break-points MIC µg/ml R |
|---|---|---|---|---|---|---|---|---|
| | Clarithromycin | 30 µg | CLARI | ≥ 18 | 17-15 | ≤ 14 | ≤ 2 | ≥ 8 |
| j) | Clindamycin | 25 µg | CLIND | ≥ 26 | 25-23 | ≤ 22 | ≤ 0.5 | ≥ 4 |
| s) | Colistin | 10 µg | CO.10 | | | | | |
| | 2+18 h prediffusion | | | ≥ 15 | 14-11 | ≤ 10 | ≤ 2 | ≥ 8 |
| v) | Daptomycin | 30 µg | DAPCa | | | | | |
| | *Staphylococcus* spp. 2+18 h prediffusion | | | ≥ 22 | - | - | ≤ 1 | - |
| | *Enterococcus faecalis* (Vanco S) 2+18h pred. | | | ≥ 12 | - | - | ≤ 4 | - |
| u) | Doripenem | 10 µg | | | | | | |
| | Enterobacteriaceae | | | ≥ 23 | - | - | ≤ 0.5 | - |
| | P.aeruginosa | | | ≥ 24 | - | - | ≤ 2 | - |
| | A.baumannii | | | ≥ 17 | - | - | ≤ 1 | - |
| | Doxycycline | 80 µg | DOXYC | ≥ 20 | 19-17 | ≤ 16 | ≤ 4 | ≥ 16 |
| q) | Enrofloxacin (Vet.) | 10 µg | ENROF | ≥ 23 | 22-17 | ≤ 16 | ≤ 0.5 | ≥ 4 |
| c) | Ertapenem | 10 µg | ERTAP | ≥ 19 | 18-16 | ≤ 15 | ≤ 2 | ≥ 8 |
| | -screen carbapenemases(Enterobacteriaceae) | | | - | - | < 22 | Test for carba-penemase | |
| j) | Erythromycin | 78 µg | ERYTR | ≥ 26 | 25-19 | ≤ 18 | ≤ 0.5 | ≥ 8 |
| a) l) | Fosfomycin (U) | 70+40 µg | FOSFO | ≥ 16 | 15-14 | ≤ 13 | ≤ 64 | ≥ 256 |
| i) | Fucidin* | 100 µg | FUCID | ≥ 28 | 27-24 | ≤ 23 | ≤ 1 | ≥ 4 |
| | Furazolidone* | 50 µg | FURAZ | ≥ 23 | 22-20 | ≤ 19 | ≤ 4 | ≥ 8 |
| q) | Gatifloxacin | 5 µg | GATIF | ≥ 18 | 17-15 | ≤ 14 | ≤ 2 | ≥ 8 |
| | *Staphylococcus* spp. | | | ≥ 23 | 22-20 | ≤ 19 | ≤ 0.5 | ≥ 2 |
| r) | Gentamicin | **40 µg** | GEN40 | ≥ 23 | 22-20 | ≤ 19 | ≤ 4 | ≥ 8 |
| f) | Gentamicin | **250 µg** | GN250 | - | - | < 14 | - | > 500 |
| | (Enterococci HLR all amino glycosides) | | | | | | | |

# EUCAST-and CLSI potency NEO-SENSITABS™

## Interpretation Zones and MIC Breakpoints according to CLSI



P. aeruginosa, Acinetobacter spp., B. cepacia, S maltophilia

PSEUDOMONAS AERUGINOSA

Zone diameter interpretative criteria and MIC breakpoints according to CLSI (formerly NCCLS) (1) when testing *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, *B. cepacia*, are listed in the tables below.

**Table 3.2-1 Interpretation for *Pseudomonas aeruginosa***

**Mueller-Hinton Agar. Inoculum McFarland 0.5. Incubation at 35 °C ± 2 degrees ambient air for 16-18 hours.**

| | NEO-SENSITABS | POTENCY | CODE | Zone diameter in mm S | I | R | Break-points MIC µg/ml S | R |
|---|---|---|---|---|---|---|---|---|
| a) | Amikacin | 30 µg | AMI30 | ≥ 17 | 16-15 | ≤ 14 | ≤ 16 | ≥ 32 |
| | Aztreonam | 30 µg | AZT30 | ≥ 22 | 21-16 | ≤ 15 | ≤ 8 | ≥ 32 |
| | Cefepime | 30 µg | FEP30 | ≥ 18 | 17-15 | ≤ 14 | ≤ 8 | ≥ 32 |
| d) | Cefepime+Clavulanate FEP+C | 30+10 µg | | detection of ESBL | | | | |
| | Ceftazidime | 30 µg | CAZ30 | ≥ 18 | 17-15 | ≤ 14 | ≤ 8 | ≥ 32 |
| d) | Ceftazidime+Clavulanate CAZ+C | 30+10µg | | detection of ESBL | | | | |
| | Ciprofloxacin | 5 µg | CIPR5 | ≥ 21 | 20-16 | ≤ 15 | ≤ 1 | ≥ 4 |
| c) | Colistin | 10 µg | Co.10 | | | | | |
| c) | 2+18 hours' prediffusion method | | | ≥ 15 | 14-11 | ≤ 10 | ≤ 2 | ≥ 8 |
| | Doripenem | 10 µg | DOR10 | ≥ 24 | - | - | ≤ 2 | - |
| | Fosfomycin | 200 µg | FO200 | ≥ 22 | 21-19 | ≤ 18 | ≤ 16 | ≥ 32 |
| | Gatifloxacin | 5 µg | GATIF | ≥ 18 | 17-15 | ≤ 14 | ≤ 2 | ≥ 8 |
| a) | Gentamicin | 10 µg | GEN10 | ≥ 15 | 14-13 | ≤ 12 | ≤ 4 | ≥ 8 |
| e) *) | Imipenem | 10 µg | IMI10 | ≥ 22 | 21-18 | ≤ 17 | ≤ 4 | ≥ 16 per 30/4 2008 |
| b) | Imipenem+EDTA | 10+750µg | IM10E | detection of metallo-ß-lactamases | | | | |
| | Levofloxacin | 5 µg | LEVOF | ≥ 17 | 16-14 | ≤ 13 | ≤ 2 | ≥ 8 |
| e) *) | Meropenem | 10 µg | MRP10 | ≥ 22 | 21-18 | ≤ 17 | ≤ 4 | ≥ 16 |
| | Minocycline | 30 µg | MIN30 | ≥ 19 | 18-15 | ≤ 14 | ≤ 4 | ≥ 16 |
| | Netilmicin | 30 µg | NET30 | ≥ 15 | 14-13 | ≤ 12 | ≤ 4 | ≥ 8 |
| | Ofloxacin (U) | 5 µg | OFL.5 | ≥ 16 | 15-13 | ≤ 12 | ≤ 2 | ≥ 8 |
| | Piperacillin | 100 µg | PIPRA | ≥ 18 | - | ≤ 17 | ≤ 64 | ≥ 128 |
| | Piperacillin+Tazobactam | 100+10µg PI+TZ | | ≥ 18 | - | ≤ 17 | ≤ 64/4 | ≤ 128/4 |
| | Tetracyclines | 30 µg | TET30 | ≥ 19 | 18-15 | ≤ 14 | ≤ 4 | ≥ 16 |
| | Ticarcillin | 75 µg | TIC75 | ≥ 15 | - | ≤ 14 | ≤ 64 | ≥ 128 |
| | Ticarcillin+Clavulanate | 75+10 µg TIM85 | | ≥ 15 | - | ≤ 14 | ≤ 64/2 | ≥ 128/2 |
| a) | Tobramycin | 10 µg | TOB10 | ≥ 15 | 14-13 | ≤ 12 | ≤ 4 | ≥ 8 |
| | Dipicolinic acid | 250 µg | D.P.A | Detection of metallo-β-beta lacta-mases | | | | |

- *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis requires incubation up to 24 hours.

*) *P. aeruginosa* low level resistant to meropenem (MIC 8 µg/ml) or Imipenem cannot be detected using the interpretation recommended by the CLSI. We recommend using Rosco's own interpretation.

Clinical isolates of *P. aeruginosa* heterogeneously resistant to carbapenems have been isolated (1). Subcolonies appearing within the zone of inhibition of Imipenem/Meropenem show higher MIC values. Automated systems and conventional agar dilution MICs using the standard $10^4$ CFU per spot inoculum may miss carbapenem-resistant mutants.
Detection of *P. aeruginosa* resistant to Colistin in University Hospital in Barcelona (reference 3).

**ArrayTube (AT) chip genotyping overview**

We apply molecular genotyping methods to monitor the *Pseudomonas aeruginosa* infection dynamics and epidemiology among CF patients. We primarily use the ArrayTube (AT) microarray from CLONDIAG to discriminate between clones isolated from infected patients. The AT technology is a DNA-chip based genotyping method in which labelled DNA is directly generated from a bacterial colony by multiplex PCR and then hybridized onto a microarray to yield a binary marker genotype (see Figure 1). We also use MLST and PFGE techniques for genotyping.

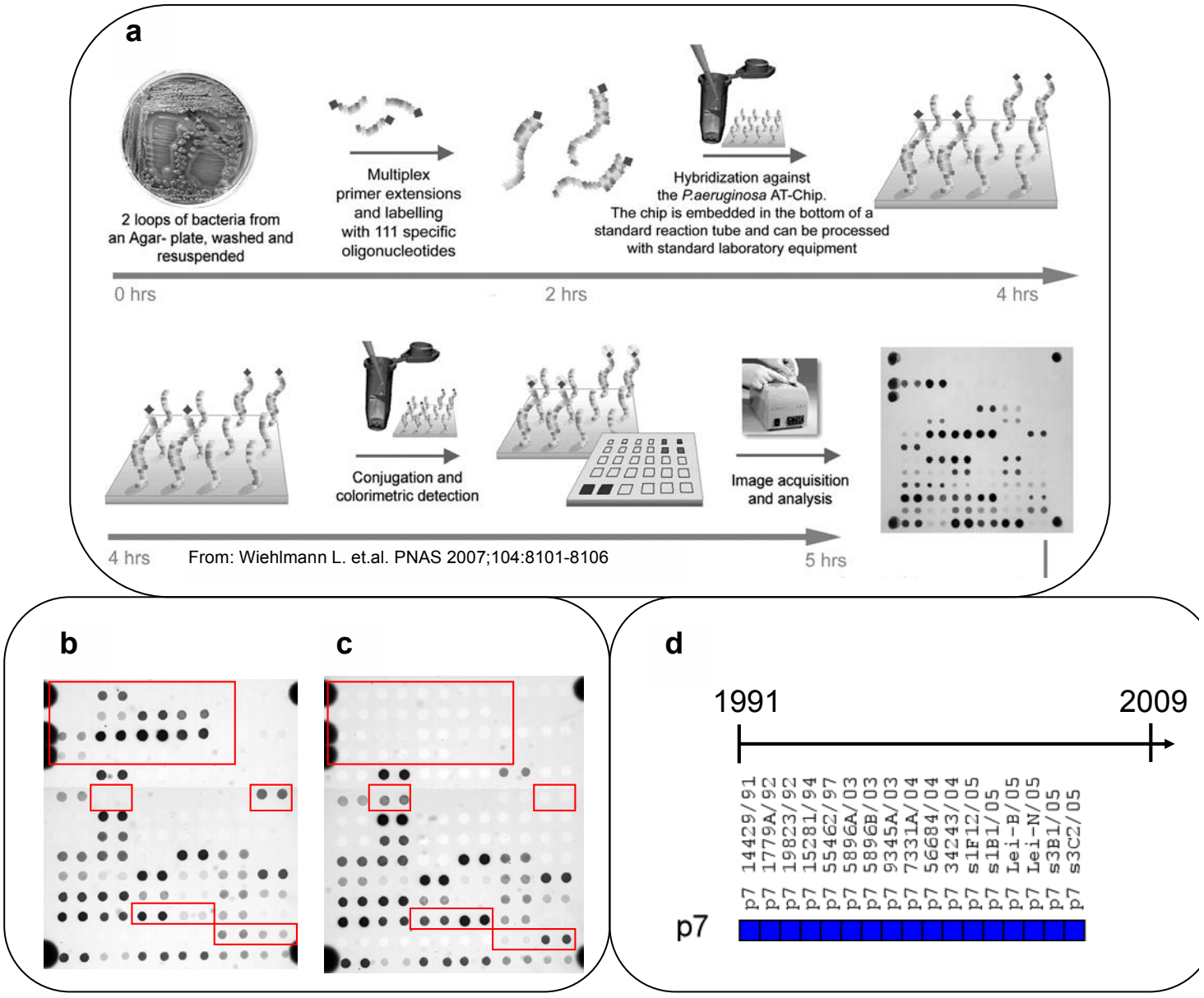



**Figure 1.** Molecular genotyping of *Pseudomonas aeruginosa*. **(a)** Outline of the experimental protocol for *P. aeruginosa* genotyping with the AT chip. **(b+c)** Examples of 2 different *P. aeruginosa* clones isolated from the same CF patient and genotyped using the AT chip. Red boxes indicate differences in the hybridization pattern. **(d)** *P. aeruginosa* infection dynamics in a single CF patient (patient "p7") with chronic *P. aeruginosa* lung infection since 1991. Genotyping of stored isolates showed that p7 had been infected with the same clone (indicated by blue squares in the figure) for the entire infection period. For details see Jelsbak et al (2007) Infect Immun. 75:2214-24.

## Solutions used in Array Tube (AT) Genotyping of *Pseudomonas aeruginosa*.

### DNA labelling and amplification

**B1Pa**: Labelling Buffer. Store at 2-8°C. Surplus: 40%.

**B2**: Labelling Enzyme. Store at 2-8°C. Surplus: 200%.

### Hybridisation and Detection

**ArrayTubes**, protected against light and sealed under inert gas. Store at 18°C to 28°C. After opening to be used within two weeks. Protect them against humidity and dust and store them at a dark place. Avoid ANY touching or scratching the microarray on the bottom of the well. CAUTION: Do not store or handle unused tubes above 60% relative humidity since this may irreversibly corrode the spots.

**C1**: Hybridisation Buffer. Store at 18-28 °C, protect against sunlight. Surplus: 100%.

**C2**: Washing Buffer 1. Store at 18 °C - 28 °C, protect against direct sunlight. Surplus: 67%.

**C3**: HRP Conjugate 100x. Store at 2-8 °C, protect against direct sunlight. Surplus: 200%.

**C4**: Conjugate Buffer. Store at 18°C to 28°C, protect against direct sunlight. Surplus: 500%

**C5**: Washing Buffer 2. Store at 18°C to 28°C, protect against direct sunlight. Surplus: 140%.

**D1**: Horseradish Peroxidase Substrate. Store at 2-8°C, protect against direct sunlight. Surplus: 140%.

## Short manual for analysis of AT chip data

1. Open the image-file of the AT chip result that you want to analyze: Open the image using e.g. 'picture-viewer'.

2. Make sure the image has the correct orientation: 3 control-spots in upper left corner (see figure 1). If the orientation is wrong it may be corrected by 'flipping' the image.

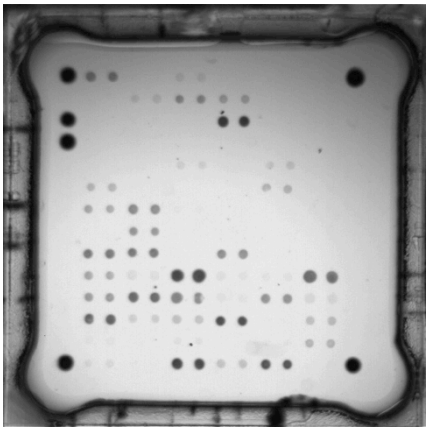


***Figure 1.*** *An ArrayTube image. Note the dark control-spots in the corners of the chip.*

3. It is a good idea to make a print-out of the image for your files.

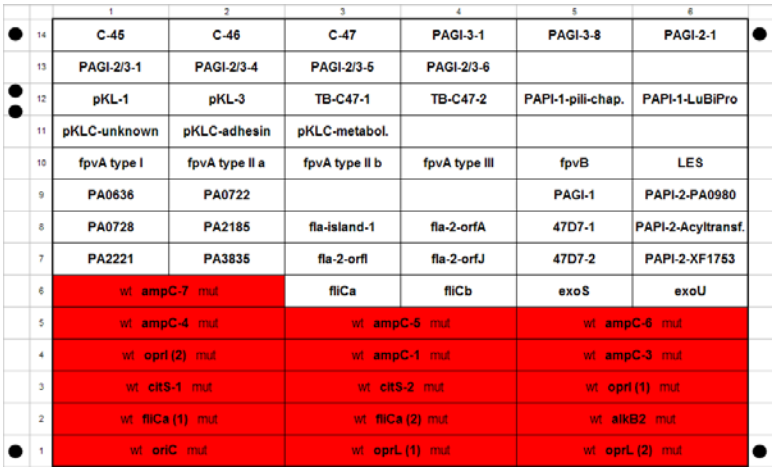
4. Print out the schematic outline of the AT chip (Figure 2 – See Figure 3 for a larger version).

5. Focus first on determining the SNP pattern. The SNP spots are found on the lower part of the AT chip (see Figure 3). With the help of the "SNP evaluation' figure (Figure 4) you can now determine whether the SNP are either 'wildtype' or 'mutant'. Record your results with a pen on the outline printout.

   *NOTE: The 3 SNPs 'oprL', fliCa' and 'oprI' are measured twice on the AT chip.*

   *NOTE: Measurement of the 'fliCa' SNP is only meaningful when there is a signal in the variable gene 'fliCa' further up on the AT chip.*

   *NOTE: If you cannot determine whether it is a mutant or a wildtype signal (maybe there is no signal at all) then note that on the outline printout.*



| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 14 | C-45 | C-46 | C-47 | PAGI-3-1 | PAGI-3-8 | PAGI-2-1 |
| 13 | PAGI-2/3-1 | PAGI-2/3-4 | PAGI-2/3-5 | PAGI-2/3-6 | | |
| 12 | pKL-1 | pKL-3 | TB-C47-1 | TB-C47-2 | PAPI-1-pili-chap. | PAPI-1-LuBiPro |
| 11 | pKLC-unknown | pKLC-adhesin | pKLC-metabol. | | | |
| 10 | fpvA type I | fpvA type II a | fpvA type II b | fpvA type III | fpvB | LES |
| 9 | PA0636 | PA0722 | | | PAGI-1 | PAPI-2-PA0980 |
| 8 | PA0728 | PA2185 | fla-island-1 | fla-2-orfA | 47D7-1 | PAPI-2-Acyltransf. |
| 7 | PA2221 | PA3835 | fla-2-orfI | fla-2-orfJ | 47D7-2 | PAPI-2-XF1753 |
| 6 | wt ampC-7 mut | | fliCa | fliCb | exoS | exoU |
| 5 | wt ampC-4 mut | | wt ampC-5 mut | | wt ampC-6 mut | |
| 4 | wt oprI (2) mut | | wt ampC-1 mut | | wt ampC-3 mut | |
| 3 | wt citS-1 mut | | wt citS-2 mut | | wt oprI (1) mut | |
| 2 | wt fliCa (1) mut | | wt fliCa (2) mut | | wt alkB2 mut | |
| 1 | wt oriC mut | | wt oprL (1) mut | | wt oprL (2) mut | |

***Figure 2.*** *A schematic outline of the AT chip. Note the control spots in the corners. The "SNP" part of the chip is highlighted in red*

6. Now you focus on the remaining spots (the variable genes). Use the outline printout and evaluate all spot. Simply record if you see a signal (This means that the gene is present). If you do no see a signal, then the gene is not there.

   *NOTE: Make sure to note any problems with the present/absent determinations*

7. Collect your data in an electronic form for further analysis

   For example, write your data into the Excel workbook called "*AT chip skabelon*". This self-explaining workbook is a good place to collect, store and analyze your data.

   Write the data into the "*Empty data sheet"* in this format:
   For the SNP data, type: '0' for wildtype, '1' for mutant, '-1' for undetermined.
   For the variable genes, type: '0' for absent and '1' for present.

   - The color of the cells in the excel sheet will change automatically.
   - An hexadecimal code will be calculated for each data-set.
   - Remember to write your notes/comments for each data-set
   - Use the "*cluster*" sheet to sort among different data sets.

**Want help?!**

1. Consult this AT chip analysis example: http://www.pnas.org/content/suppl/2007/04/18/0609213104.DC1/09213SuppAppendix.pdf
2. This paper describes the development and use of the ArrayTube genotyping method for *P. aeruginosa*: *Wiehlmann et al. (2007) Population structure of Pseudomonas aeruginosa. PNAS 104(19):8101-6.* http://www.pnas.org/content/104/19/8101.full.pdf